3-METHYLCHOLANTHRENE CONCENTRATION AND CLEARANCE IN SOME ADIPOSE TISSUES IN MICE

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INTRODUCTION

Three hundred years ago, the role of chemical compounds in carcinogenesis was first suspected and investigated by Percival Pott, a surgeon to St. Bartholomew's Hospital, who found an increased incidence of cancer of the scrotum in chimney sweeps and traced it to soot contamination of the scrotal skin. Hundreds of experiments and thousands of experimental animals later, the picture is infinitely more complex. Now carcinogens are reported virtually monthly that produce a variety of tumors in different species of animals, when administered at different dosages and by different routes of administration. Theories of mechanisms of action of carcinogens are necessarily speculative and at best apply only to a small percentage of cases. Old concepts are supplanted by newer ones that are in turn replaced by the former, re-thought and re-evaluated in the light of current data. As a result, one remains unconvinced by any and suspicious of all. The solution, then, to the central problem of how normal cells become malignant is still remote, and the amount of work to be done is massive.

The study of carcinogenesis is properly the study of carcinogens. It seems necessary therefore to attempt to characterize these carcinogenic compounds, especially with regard to the animal tissues where they localize and to estimate as closely as possible the amounts that are absorbed intact into susceptible tissues. The purpose of this paper is to present findings concerning the concentration and clearance of the carcinogen.

3-methlycholanthrene (3-MC) in selected adipose tissues of mice, and to examine chemical carcinogens as a whole and their special ability to induce mammary carcinoma, to evaluate the factors influencing the induction of mammary cancer, and to discuss the theories proposed for the mechanism of carcinogenesis.

HISTORICAL ASPECTS

After Pott reported his discoveries on scrotal cancer in Chirugical Observations in 1775, little was heard of what came to be known as chemical carcinogenesis until 1875 when occupational skin tumors were found by von Volkman among workers in the tar and paraffin industry. The following year Joseph Bell of Edinburgh described the earliest cases of "paraffin cancer" in the Scottish shalefields. Later in 1887 "mule-spinners cancer" was reported, a skin tumor which affected workers in the Lancashire cotton-spining industry and was apparently caused by contact with mineral oil. Following these undesigned and unpredictable natural experiments, systematic inquiry was begun. The first malignant epithelial tumors were induced in 1917 by Yamagiwa and Ichikawa who applied coaltar to the ears of rabbits. Passey then proved the carcinogenicity of soot in 1922 by producing malignant growths after painting mouse skin with an ethereal extract (27).

NATURE OF COALTAR AND PITCH

Naturally enough, a search for the "active ingredient" in coaltar rapidly became the subject of much investigation. In 1920 the Schroeter reaction was developed in which a number of complex compounds were produced by treating tetralin (tetrahydronaphthalene) with a 1-15% solution of

aluminum chloride at 35-40°C. Kennaway tested a mixture of these compounds and induced 110 cancers in 496 mice. The Schroeter compounds demonstrated a vivid blue-violet fluorescence in ultra violet and ordinary daylight. In 1926, Mayneord examined the Schroeter preparation with a spectrophometer and found two very distinct bands in the blue and violet regions. He then photographed carcinogenic and noncarcinogenic compounds and found the Schroeter preparation bands to be in the carcinogenic group. As Hieger says, Mayneord "made the fundamental observation--namely, that the same characteristic bands were to be found in the spectra of tars and other carcinogenic substances such as the 'Schroeter mixture'." The next year Hieger reported that 1,2-Benzanthracene had a spectrum very similar to the Schroeter preparation. In 1929, Erich Clar developed the method for synthesizing certain slightly more complex hydrocarbons containing the benzanthracene system. These compounds were tested for carcinogenicity by Kennaway and Hieger, and positive results were obtained with 1,2,5,6-dibenzanthracene, the first pure chemical compound known with carcinogenic properties. In 1933, a pure hydrocarbon was isolated by Hieger with fluorescence spectroscopy from a concentration of the carcinogenic agent in coaltar pitch and found it to have a three-banded spectrum and to be actively carcinogenic. It was identified by Cook and Hewett as 3,4-benzpyrene and was subsequently synthesized (36).

During this same period significant advances in sterol formation were being made by Rosenblum and King, who found that many naturally-occurring compounds, including bile acids and sex hormones, have condensed polycyclic systems similar to carcinogens. For example, dehydronorcholene, a hydrocarbon prepared by Wielland and Schlichting from deoxycholic acid is a hydro derivative of 1,2-benzanthracene. From it, Wielland and Dane,

and Cook and Haslewood prepared by dehydrogenation with selenium the fully aromatic hydrocarbon, methylcholanthrene, which proved to be a highly potent carcinogen (27).

RELATION TO MAMMARY CANCER

It appears somewhat accidental that polycyclic hydrocarbons were ultimately found to produce mammary cancer. The early experiments were set up to investigate skin cancer produced by these substances in non-inbred strains of mice with an unknown spontaneous incidence of mammary cancer. To the surprise of all concerned, these compounds regularly induced breast tumors either simultaneously with or instead of skin cancer. The most efficient carcinogen in this respect has been methylcholanthrene.

The significant experiments in which 3-MC was used as the carcinogen have been tabulated chronologically and include species of animal, age, dose, route of administration and results.

METHODS OF LOCALIZATION

Methods for localizing polycyclic hydrocarbons in the tissues of experimental animals have received close attention in order to determine the specific target organs of these compounds. Naturally enough, advantage has been taken of their fluorescence in ultraviolet and ordinary daylight, and several methods were devised using this quality.

Doniach, Mottram and Wiegert (20) in 1943 studied the fluorescence in mouse skin and other organs with a glass spectrograph. Immediately after painting mouse skin with benzpyrene (BP) in an acetone or benzene solution or in a watery suspension, they found that it fluoresced a brilliant violet which in a few days changed to blue. The violet spectrum

TABLE 1

Comparison of Experiments Using Chiefly 3-MC as Carcinogen with Respect to Animal, Age, Dose, Route of Administration and Results

Author	Date	Species	Sex	Age	Carcinog.	Dose	Route	Results
Maisin & Coolen (46)	1936	Non-inbred	♀=F	-	MC		Skin-paint	29% MCa.†
		"	"	-	BP		" "	32% "
Perry & Ginzton (56)	1937		♀	adol.	DBA	0.3% benzene	" " 2x/wk.	0/25 MCa.
			"	"	DBA + Theelin	" " 0.1%	" "	6/14(43%)MCa.
			Castrated ♀*	"	DBA	0.3% "	" "	4/37(11%)MCa.
			"	"	DBA + Theelin	" " 0.1% "	" "	7/15(43%)MCa.
Bonser & Orr (9)	1939	IF	♀	-	MC	lard solution	Subcut. injection	4 MCa. 8 Sarcomes + MCa.
		White label	"	-	"	" "	"	1 MCa. 1 Sarcoma + MCa.
		Bagg albino	"	-	"	" "	"	1 " + "
		CBA	"	-	"	" "	"	2 Sarcomes + MCa.
		Outbred lab stock	"	-	"	paraffin pel.	Subcut. implant	2 MCa.
		" "	"	-	DBA	" "	"	2 MCa. 1 Sarcoma + MCa.
Mider & Morton (50)	1939	dba #212	breed ♀	-	MC	0.5+0.25% benzene +0.25% acetone	Skin-paint	13/65
		"	virg. ♀	-	"	"	" "	0/75
Strong & Smith (63)	1939	NH,HE,CBA,N, JK, C_{57},CBA	♀	2 mos.	"	1 mg. sesame oil	Subcut. injection	18/42 Sarcomes + 8/42 MCa. (6=NH) (2=JK) 16/42 Sarcomes
		"	♂	" "	"	"	"	

* Mice unless otherwise noted; † Mammary cancer.

Author	Date	Species	Sex	Age	Carcinog.	Dose	Route	Results
Bonser (8)	1940	IF	♀	9-16 wks.	MC	1.0 mg. lard	Subcut. injection	12/33 MCa.
		"	♂	" "	"	" " "	" "	0 "
		Bagg albino	♀	9-20 "	"	" " "	" "	1/16 "
		" "	♂	" "	"	" " "	" "	0 "
		CBA	♀	6-9 "	"	" " "	" "	2/12 "
		"	♂	" "	"	" " "	" "	0 "
		White label	♀	9-20 "	"	" " "	" "	2/11 "
		" "	♂	" "	"	" " "	" "	0 "
		Market	♀	-	"	" " "	" "	0 "
		"	♂	-		" " "	" "	0 "
Engelbreth, Holm (21)	1941	dba	♀	4-6 wks.	"	0.5% benzene	Skin-paint 1x/wk	19/27 MCa.
		"	♂	" "	"	" "	" " "	0 "
Engelbreth, Holm & Lefevre (22)	1941	Sku	♀	4-8 mos.	DMBA	" "	" " 2-3x/wk.	10/14 leukemia
		Dlb	"	"	"	" "	" " "	7/50 MCa.
		"	"	"	"	0.5-1.0 mg. olive oil	Subcut.	3/44 MCa.
Strong & Williams (64)	1941	NH	breed ♀	60 days	MC	1 mg./0.10 sesame oil	"	45/384 MCa.
		"	♂	"	"	"	"	0 "
Kirschbaum & Strong (40)	1942	F	breed ♀	6 wks.	"	horse serum	IV injection	3/6 MCa.
		"	♂	"	"	" "	" "	0 "
Orr (55)	1943	IF	♀	-	"	2cc of sweet almond oil	Intranasally	69% MCa.
		"	♂	-	"	oil solution	every 2 wks.	0 "
		CBS	♀	-	"	"	"	67% MCa.
		"	♂	-	"	"	"	4% "
		IF	♂	-	"	+ estrogen	"	69% "

TABLE 1, (cont'd)

Author	Date	Species	Sex	Age	Carcinog.	Dose	Route	Results
Kirschbaum et al. (39)	1944	DBA	breed ♀	6-8 wks	MC	o.5% benzene	per cut 2x/wk	27/27 MCa. (at 105d)
		"	" "	"	No treatment			23/27 MCa. (at 370d)
		"	virg. ♀	"	MC	0.5% benzene	per cut 2x/wk	4/31 MCa.
		"	o→	"	"	" "	"	0/48 "
Kirschbaum & Bittner (38)	1945	C_3H ♀ x dba o→	breed ♀	$\bar{c}$ MTA -	"	0.25% "	skin-paint 2-3x/wk	5/6 "
		"	" "	$\bar{o}$ " -	"	" "	"	1/16 "
		(C_3H♀ xdba o→) ♀ x dba o→	" "	$\bar{o}$ " -	"	" "	"	5/15 "
		dba	" "	$\bar{o}$ $\bar{c}$ " -	"	" "	"	27/27 "
		C_3H ♀ x dba o→	virg. "	$\bar{c}$ " -	"	" "	"	1/7 "
		"	"	$\bar{o}$ " -	"	" "	"	0/28 "
		dba	" ♀	$\bar{c}$ " -	"	" "	"	4/31 "
		C_3H	" ♀	$\bar{c}$ " -	"	" "	"	0/12 "
Strong (62)	1945	NHO	♀	-	"	1 mg./0.1 cc sesame oil	subcut.	60% "
Kirschbaum et al. (41)	1946	dba	breed ♀	5-8 wks	"	0.25% benzene	skin-paint 3x/wk	27/70 MCa.
		"	virg. "	"	"	" "	"	7/70 "
		A	breed "	"	"	" "	"	29/39 "
		Z($\bar{c}$MTA)	" "	"	"	" "	"	15/40 "
		"	virg. "	"	"	" "	"	1/40 "
		Zb($\bar{o}$MTA)	breed "	"	"	" "	"	11/38 "
		"	virg. "	"	"	" "	"	1/38 "
		NH	breed "	"	"	" "	"	10/27 "
		C_{57}	"	"	"	" "	"	0 "
		F	"	"	"	" "	"	0 "

TABLE 1, (cont'd)

Author	Date	Species	Sex	Age	Carcinogen	Dose	Route	Results
Kirschbaum (37)	1949	NH♀x(NH♀xDBA♂)♂ breed	♀	-	MC	0.25% benzene	skin-paint 3xwk	3/18 MCa.
		NHDxNHDF$_2$	"	-	"	" "	"	3/15 "
		NHDxD backcross	"	-	"	" "	"	2/22 "
Shay, et al. (60)	1949	Wistar rat	"	5-6 wks to 2 yr.	"	2 mg./0.5 cc olive oil	intragastric 6d/wk	50/70% MCa.
		" "	♂	"	"	"	"	2/19 "
		" "	♀	"	"	"	"	0/20 "
		" "	♂	"	"	"	"	1/20 "
Andervont & Dunn (1)	1953	Dbaf/2	♀	-	"	1 mg./0.2 cc olive oil	force fed	32% "
		"	"	-	"	+sh/bestrol (0.5 mg)oil	" "	55% "
		"	"	-	"	+ " (1.0 mg) "	" "	41% "
		"	♂	-	"	1 mg./0.22 cc olive oil	" "	0 "
Huggins, et al. (34)	1959	Sprague-Dawley rats	♀	6 wks.	"	10 mg./d	intragastric 3-6x/wk	100% "

TABLE 1, (cont'd)

showed three peaks, the main one at 427 mu and weaker ones at 405 and 455 mu, corresponding to that exhibited by the molecular dispersion of BP. The blue also had two peaks: 450 mu and 425 mu, similar to that of orthohombic crystals of BP. After innoculation of BP into mice and rabbits, the fluorescence of kidney, lung and liver slowly changed from violet to blue in 2-4 hours, while the milk of lactating mice showed blue in 4 hours. In addition, they found that only the kidney cortex was blue, and that in skin, the most strongly fluorescent area was the rows of hair bulbs.

That same year Simpson and Cramer (61) used fluorescence microscopy to investigate the localization of MC in mouse skin. Dry MC gives a yellow-green fluorescence in ultraviolet light, which in benzene, acetone, lard or anhydrous lanolin it produces a strong blue-violet color. They noted that immediately after application, the bulk of the MC was found in the epidermis at two sites: the sebaceous glands and the keratin layer, and felt that it was dissolved in the sebum and free lipids of keratinized epithelium. Subsequent changes involved the degeneration and disappearance of sebaceous gland cells with much excretion of sebum with MC from the glands to hair follicles. From the follicles it moved to the keratin surface layer and was followed by epilation and the gradual flaking off of keratin soaked with sebum and MC. There was no evidence that MC could be taken up by the epithelial cell directly. Fat cells in the subcutaneous tissues got some MC, but lost it readily. After 6-10 days, all fluorescence had disappeared.

Dao, Bock and Crouch (15) described a macroscopic method using fluorescence for the quantitative determination of the location and level of MC in various tissues of rats after intragastric administration.

After sacrifice, the tissues were weighed, minced and treated with equal volumes of 95% ethyl alcohol and 10% potassium hydroxide, refluxed for 6-8 hours, and extracted twice with benzene. The pooled fractions were read on a spectrophotofluoremeter and were considered to represent 85-95% recovery. They found that after 24 hours, the MC levels were highest in the breast and fat tissues, and decreased rapidly after 72 hours, but were still significant after 8 days. Levels of the carcinogen in other organs were extremely low compared with breast and fat. This procedure was used exclusively in the present experiments.

Such methods involving natural fluorescence were employed almost exclusively until Borum (10) in 1961 used radioautography to localize single paintings of carbon-14 labelled dimethylbenzanthracene (DMBA) on mouse skin. He found that the radioactivity localized in the epidermis, appendages and deeper connective tissues and muscle, persisted for several days, and did not vary from resting to growth phases. The hamster cheek pouch, however, has no appendagal structures, and in 1964, Meskin and Woolfrey (49) described an experiment in which carbon -14 labelled DMBA was applied on one side of the pouch and unlabelled carcinogen was applied to the opposite side. After fixing and developing the specimens, they found that at zero time there was marked localization on the surface of the mucous membrane with a distributed intensity throughout the stratified squamous epithelium and subjacent loose connective tissues. During the next 4 hours, there was progressive loss from the free surface and stratified squamous epithelium with a slight decrease in the submucosal zone. The epithelial radioactivity dropped to slightly above background during the following 8-12 hours, while the submucosa retained a patchy distribution with slight localization around the muscle bundles. The

surface epithelium and mucosa both returned to background in 24-48 hours, but the submucosa remained radioactive in spots until 48 hours had elapsed. These workers felt that carbon-14 labelling adds only slightly to the resolution obtained with fluorescent techniques, but that tritium labelling should improve it.

The problem of the preparation and purification of tritiated hydrocarbons is a difficult one, and the standard procedure devised by Wilzbach in which the hydrocarbon is exposed to high levels of tritium gas produces many impurities. However, Giovanella, Abell and Heidelberger (24) used column and paper chromatography for purification with considerable success. Using their revised method, Heidelberger and Abell conducted studies comparing the binding of carcinogenic and non-carcinogenic compounds to the soluble proteins of the skin of female Swiss albino mice. They found a quantitative correlation between binding to Protein fraction I (which migrated toward the cathode with a relative mobility of +0.23 in starch gel electrophoresis) and the carcinogenicity of the hydrocarbon. Carcinogens were also bound considerably to Protein fraction II (which migrated toward the anode with a relative mobility of -0.77), much more extensively than to noncarcinogens which were bound to all protein fractions except I and II.

EXPERIMENTAL

Material and Methods

In three of the four experiments reported in this paper, 135 female C57BL/6J mice, 6-7 weeks old, received a single intraperitoneal injection of 3-MC dissolved in sesame oil. Two sets of controls were used, one receiving no treatment, the other given injections intraperitoneally of 1 ml. of sesame oil and sacrificed 9 hours after injection. In Experiment I the mice received 1, 5, 10 or 20 mg. of 3-MC and were sacrificed 7 hours after injection. In Experiment II the mice received 30 mg. of 3-MC and were examined 1, 3, 5, 6, 7, 8, 9, 12, 15, 18, and 24 hours after injection. The following tissues were removed: in I, the third mammary gland fat pads, the fourth mammary gland fat pads and the interscapular brown fat pad; in II, the fourth mammary gland fat pads and the interscapular brown fat pad. The brown fat pads were cleared as closely as possible of the surrounding white fat. Segments of the third mammary fat pads were removed ventro-medially from the extreme dorsal end to the arbitrary border of the fifth mammary fat pad. In Experiment III, 31 of 47 fourth mammary fat pads, which were removed at 9 hours after injection of 30 mg. of 3-MC, were transplanted into the subcutaneous areas of 31 intact female host mice of the same strain and age. Transplants were removed from the hosts at 3, 6, 9 and 15 hours and 3, 7, and 10 days after the time of transplantation. In Experiment IV 48 female (BALB/c X C3H/He) F_1 hybrid mice, 3 weeks old, were operated on to remove the mammary parenchyma from the right mammary gland tissues by the type E operation (33). At 4-8 weeks of age they received, intraperitoneally, 10 mg. of 3MC in 0.5 ml. of sesame oil and were sacrificed 7 hours after the single injection. The right fourth mammary gland-free fat pads were totally removed for assay. The

left fourth mammary fat pads were partially excised from the areas adjacent to the nipples including the ventral inguinal lymph nodes.

All specimens were weighed, minced, hydrolyzed, and refluxed with alcoholic potassium hydroxide, and extracted with fluorescence-free benzene. Two to 6 fat pads were pooled before extraction. One or three benzene extracts for each group of determinations were examined with the Aminco-Bowman Spectrophotofluorometer using the 295 mu excitation light and measuring the 410 mu emission peak with slit width arrangement No. 5 having 3/16th inch of cell slit at positions of Nos. 2, 3 and 5. This procedure is a modification of the method described by Dao et al. (15). The accuracy of the method was tested by adding 5 micrograms of 3-MC in 0.05 ml. of sesame oil to the extraction system. Recovery in six determinations ranged from 4.8 to 5.2 micrograms (average 5.1 micrograms).

Results

Detailed results are presented in the four accompanying tables.

For dosages of 5, 10 or 20 mg. of 3-MC the following consistent relationship in 3-MC concentrations among the three different adipose tissues was observed: fourth mammary fat pad < third mammary fat pad < interscapular brown fat. (Table 2) No remarkable differences existed between the third mammary fat pad and the brown fat pad for dosages of 1 and 5 mg., but both were greater than the controls. Concentration of 3MC in each of the three different adipose tissues increased proportionately with increasing dosage, but in a nonlinear fashion.

In the experiment of group II the concentration of 3-MC for each group of determinations was always greater in the brown fat than in the white fat of the mammary fat pads. The peak concentration for both tissues

after injection of 30 mg. of 3MC occurred by 10 hours and leveled off after 12 hours. (Table 3)

The concentration of 3-MC in the transplanted tissues in the experiment of group III ranged between 4.6 and 6.3 micrograms/g of tissue until 15 hours after transplantation, but by 10 days had gradually declined to 0.4 microgram/g of tissue, which was still slightly higher than the control values. (Table 4)

In the last experiment, 3-MC concentration was greater in the fourth mammary fat pads including the parenchyma than in those which were gland-free. (Table 5)

TABLE 2

Comparison of 3-Methylcholanthrene Concentration in Three Different Adipose Tissues in Mice 7 Hours after Intraperitoneal Injection of Various Dosages of 3-MC in Sesame Oil

3-MC (mg.)/ml Sesame Oil	3-MC concentration (μg.)/gm tissue assayed*		
	(1) (μg/gm)	(2) (μg/gm)	(3) (μg/gm)
0/0	0.1	0.2	0.3
0/1 ml	0.2	0.4	0.1
1 mg/1	0.3	1.0	1.0
5/1	0.5	1.0	1.1
10/1	1.0	1.3	2.4
20/1	3.0	3.6	6.5

*In each determination six fat pads were pooled, extracted, and assayed. All determinations were done at the same time.

Note: Range of weights of tissues assayed: (1) the fourth mammary gland fat pads, 635 ~ 1030 mg. (2) the third mammary gland fat pads, 200 ~ 505 mg. (3) the interscapular brown fat pads, 150 ~ 190 mg.

Body weight of mice ranged from 16.0 to 20.5 gm.

TABLE 3

3-MC Concentration in Mammary Fat Pads and Brown Fat Pads in Mice Receiving Single Intraperitoneal Injections of 30 Mg. of 3-MC in Sesame Oil

Hours after injection	Fourth Mammary Fat Pads			Interscapular Brown Fat Pads		
	Tissue assayed		3-MC (μg.) per gram tissue	Tissue assayed		3-MC (μg.) per gram tissue
	No. pads	Total weight (mg.)		No. pads	Total weight (mg.)	
1	6	695	1.6	6	200	1.7
3	10	1205	2.4 (1.7,3.0)	10	310	7.9 (7.6,8.1)
5	10	1050	4.3 (3.9,4.7)	10	277	15.7 (2.5,28.9)
6	12	1455	6.6 (4.4,8.8)	12	353	12.3 (4.9,19.6)
7	16	1985	5.2 (3.1,4.0,8.6)	16	490	15.2 (9.0,16.2,20.5)
8	12	1375	9.8 (5.1,14.5)	12	349	12.0 (7.9,16.0)
9	16	1325	7.3 (6.0,6.1,9.7)	10	240	16.0 (5.3,13.6,29.1)
10	12	1015	11.6 (4.2,19.0)	12	305	34.3 (10.0,58.7)
12	10	855	4.8 (2.2,7.5)	4	90	13.6
15	10	1233	4.0 (3.4,4.6)	10	315	6.4 (2.7,10.0)
18	16	1788	5.3 (1.0, 4.0,10.8)	16	450	9.0 (1.1,5.3,20.7)
24	10	670	4.6 (4.2,5.1)	10	245	9.4 (8.2,10.5)
Controls:						
1 ml. sesame oil alone	6	915	0.2	6	185	0.1
No injection	6	1030	0.1	6	190	0.3

Note: The average values are presented, followed by the individual values in parentheses. Body weights of experimental animals ranged from 16.5 to 20.1 gm.

TABLE 4

3-Methylcholanthrene Concentration in Mammary Fat Pads Transplanted Subcutaneously from Mice That Had Been Pretreated with 30 Mg. of 3-MC 9 Hours before Donation of Tissues

Time after Transplantation	Tissue Assayed		3-MC Concentration (μg.) per gram tissue
	No. pads	Total weight (mg.)	
0 hour	16	1325	7.3 (6.0, 6.1, 9.7)
3	5	720	4.6 (2.6, 6.5)
6	6	645	6.3 (4.8, 7.9)
9	6	660	5.9 (4.7, 7.0)
15	5	585	5.7 (2.8, 8.6)
3 days	3	540	1.6
7	3	550	0.7
10	3	475	0.4

Note: Body weights of donor and host mice ranged from 16.2 to 18.0 gm. The average values are presented, followed by the individual values in parentheses.

TABLE 5

Comparison of 3-Methylcholanthrene Concentration in the Fourth Mammary Gland-free Fat Pads and the Fourth Mammary Fat Pads Including Mammary Parenchyma in Young Mice

Group	Fourth Mammary Gland-free Fat Pads		Fourth Mammary Fat Pads Having Mammary Parenchyma	
	Weight of tissue assayed (mg.)	3-MC conc. (μg.) gm tissue	Weight of tissue assayed (mg.)	3-MC conc. (μg.)/gm tissue
1	22	3.7	21	11.6
2	15	2.7	17	4.8
3	47	8.5	40	15.0
4	29	6.9	29	17.9
5	34	3.6	41	4.0
6	190	8.2	140	7.9
7	240	5.2	185	5.4
8	140	9.3	120	17.8
9	80	15.3	60	17.7
10	120	5.8	100	11.2
11	210	8.1	120	19.6
12	180	13.6	100	15.4
Mean standard error:		7.6 ± 1.1		12.4 ± 1.6

Note: Forty-eight 3-week-old female mice were operated on to make the right fourth mammary glands free of mammery parenchyma. When they were 4-8 weeks of age they were divided into twelve groups and received 10 mg. of 3-MC in 0.5 ml. of sesame oil intraperitoneally. Seven hours after single injections, fat pads were removed from either side of the fourth mammary tissues. Four fat pads from the same side were pooled and assayed for each determination. The difference between two means is statistically significant ($P < 0.025$).

DISCUSSION

The importance of the solvent in chemical carcinogenesis has been emphasized both with respect to tumor induction and the concentration of the carcinogen in the target organs. Vegetable oils were the most efficient vehicle (6, 16, 18). Dao and his collaborators (16) failed, even with 100 mg. doses of 3-MC in aqueous suspension, to produce tumors and to detect 3-MC in breast or fat tissues by the third day after a single feeding in rats. The selective concentration and extended period of retention of unchanged 3-MC in adipose tissue, when administered in lipid solution, remains unexplained. The greater affinity of 3-MC for the interscapular brown fat as compared with the white fat is also unexplained.

The patterns of clearance of 3-MC from the tissues in situ and from the transplanted tissues were different. The 3-MC concentration 3 hours after transplantation was low in comparison with that in the 6- to 15-hour transplants which were, in turn, higher than in the corresponding non-transplanted tissues. Instead of being cleared from the grafts to the host's body which was free of 3-MC, the carcinogen was retained in the transplanted tissues in a relatively high concentration until 15 hours after transplantation. Subsequent clearance of 3-MC from the grafts was also slow. However, the pattern of carcinogen clearance from the transplanted tissues was comparable to that from rat breast and adipose tissue in situ after a single feeding of 3-MC reported by Dao et al. (6, 15, 16). It remains to be seen whether the quantity of 3-MC retained in the transplanted mammary tissues is sufficient to initiate carcinogenesis in these tissues when transplanted in host animals.

Since the amount of mammary parenchyma removed with the third mammary fat pads was proportionately larger than that removed with the

fourth by the technic employed in this experiment, and in view of the fact that 3-MC concentration in the third mammary fat pads was higher than in the fourth (Table 2), it was suspected that 3-MC has a greater affinity for mammary parenchyma than for mammary fat. This finding, however, is opposed to the conclusion drawn by Bock and Dao (6), who presented data obtained 2 hours after intragastric instillation of 3-MC in a single rat. Their technic involved the isolation of mammary tissues from the mammary fat, a difficult procedure in nonpregnant rats. The exact amount of fat in the tissue removed was not determined. These facts were also supported by Hoshino (32, 33). In view of this, and because some amounts of 3-MC must be lost during microdissection from the mammary tissues to be analyzed, the technic described above was used, and contradictory results were obtained. The rapid and selective development of mammary tumors after remote administration of polycyclic hydrocarbons (17, 34, 47) may be partly due to the great affinity of 3-MC for mammary parenchyma as well as for the surrounding fat pads.

Few attempts have been made to localize carcingoenic hydrocarbons in animal tissues.

In 1936 Hieger (30) reported the use of ultraviolet light to localize grossly the retention of fluorescence on the surface of mouse skin after the application of carcinogens. Beck and Peacock (3) used the same technique in 1940 with benzpyrene.

The experiments conducted by Doniach, Mottram and Wiegert, and Simpson and Cramer, and Borum with mouse skin; of Meskin and Woolfrey with hamster cheek pouch; and of Dao, Bock and Crouch with rat tissues have already been discussed under the section dealing with localization methods.

In 1943 Perry, Strait and McCawley (57) noting that protracted

administration of estrogens to mice induced mammary carcinoma, and using a spectrochemical method, investigated the relationship between injected estrogen and mammary tissue at a time preceding and following tumor formation. They injected the synthetic estrogen triphenylethylene subcutaneously into male and female C_3H mice until mammary tumors developed, then excised the tumors and remaining mammary tissue (considered to be precancerous), and after extracting with ether, analyzed them with a single beam, Baly tube, and densitometer to determine the quantity of estrogen present. They found no estrogen in the tumors, but noted significant levels of triphenylethylene in the precancerous mammary glands up to 7 days after injection. The glands of males contained more estrogen than those of females, but developed no tumors. The mammary glands from mice injected only once with the estrogen contained less triphenylethylene and retained it for shorter periods of time than the precancerous glands. The authors speculate that the changing ability of the tissue to accumulate estrogen may be connected with the initiation of malignancy.

FACTORS INFLUENCING MAMMARY CANCER INDUCTION

Mammary Tumor Agent (MTA)

Lathrop and Loeb (44) first noted a maternal influence in the development of mammary cancer in hybrid mice, and Korteweg (42, 43) and Murray and Little (54) showed that this influence does not take place through the chromosomes. Bittner (7) then found that the milk of high-mammary-cancer-strain mice transmitted the tendency for cancer to their young by suckling.

3-MC induces mammary cancer in low-cancer-strain mice and accelerates its development in other high-cancer-strain mice. Estrogenic hormones are required because only females develop tumors and breeding females show a higher incidence than virgin mice. According to Dmochowski (19):

> The development of breast tumors in high and low-cancer-strain mice following treatment with methylcholanthrene, the differences in susceptibility to the development of tumors in various high-cancer-strain as well as between low-cancer-strain, and the absence of the agent in induced breast tumors of low-cancer-strain mice support the conclusion that the action of methylcholanthrene is [illegible] independent of the milk agent. This conclusion strengthens the opinion [of Strong, 1945 (62)] that the type of susceptibility involved in the origin of carcinogen- induced tumors differs from that involved in the origin of spontaneous breast cancer, all the more as mice with low susceptibility to the milk agent may show high susceptibility to methylcholanthrene and vice versa. Methylcholanthrene does not induce the appearance of the agent in mice which does not possess it, and while few or no breast tumors develop in agent-free, even susceptible mice following treatment with estrogens, methylcholanthrene induces breast cancers in agent-free mice, both susceptible and resistant to the milk agent.

Hormones

The influence of hormones on the development of mammary cancer can best be evaluated in mice without the MTA. Many investigators have found that only breeding females regularly produce tumors and that virgins rarely if ever develop them. In addition, an increase is the number of

pregnancies increases the tumor incidence on agent-free mice. Since the growth and function of the mammary gland depend on hormones from the pituitary and ovary and increase tremendously in pregnancy, it would seem that hormones alone would have a carcinogenic effect (53). According to Gardner (23):

> ...Administration of suitable amounts of all the estrogenic chemicals have been followed by mammary cancers in mice of suitable strains, and when the mammary tumor virus or agent is present. ...Mice given very large doses of estrogen show less extensive mammary growth and fewer tumors than those given smaller amounts. ...Estrogens do no more than do the intrinsic hormones of multiparous females in their contribution to mammary carcinogenesis in the mouse.

Estrogens are less effective in inducing breast cancer in mice without the MTA. Female mice given testosterone propionate have fewer tumors than untreated mice, and castration generally reduces or prevents mammary carcinoma in females. In addition, carcinogenic hydrocarbons increase mammary cancer incidence in agent-free mice, but only in females with mammary growth influenced by hormones, whether intrinsic or exogenous.

The effect of pituitary hormone has also been studied. Loeb and Kirtz (45) showed in 1929 that by subcutaneous transplantation of hypophyses into virgin animals of the A strain with MTA, the tumor incidence was increased. Mühlbock (53) transplanted pituitaries (5/week for a year) subcutaneously into C3Hf, DBAf, O_{20}, and C47Bl, all without MTA and got 60-80% tumors in all except the last. At no time could the MTA be detected in any of the tumors, so that it appears that the pregnancy factor in mammary cancer is actually a hormonal one. In any case, hormones are necessary for the development of mammary cancer if only in providing the substrate for tumors--the mammary gland.

MECHANISMS OF ACTION

Initiation and Promotion

The view that carcinogenesis is a multi-stage process was first proposed, independently but almost simultaneously, by Rous (59) and by Berenblum (4). Initially Berenblum postulated 3 stages for the production of cancer, namely, pre-, epi- and meta-carcinogenesis, but now seems content with Rous' two: initiation and promotion. The large and ever-expanding numbers of carcinogens led these investigators to wonder whether they could be used interchangeably, and if the process could be started by one carcinogen and be completed by a totally different compound. Rous found that tumors produced by tarring rabbits' ears, which regressed when tarring was stopped, could be made to reappear by retarring or by application of non-carcinogenic stimuli such as wound-healing, turpentine or chloroform. The first tarring he termed Initiation and the subsequent treatment, Promotion. During initiation, normal cells are converted into "latent" tumor cells, while the promoting action allowed them to evolve into visible tumors. Berenblum supported Rous' findings with his study of cocarcinogenic action of croton oil on the skin of the mouse. He reported that application of a single threshold dose of benzpyrene (0.05%) followed by croton oil results in approximately the same number of skin tumors as 1.0% BP (the usual tumor dose), while 0.05% BP alone and croton oil alone elicited no tumors. According to Berenblum (4):

> (1)...Initiation is an irreversible process; and (2)...the number of tumours eventually appearing is predetermined by the potency of the initiating stimulus, while the speed with which they appear is dependent on the effectiveness of the promoting action.

Hieger (31) notes that the results of Rous and Berenblum are in excellent agreement and adds further generalizations based on their later work:

> ...(1) the initiator must necessarily be a carcinogen; (2) it can be employed at threshold intensity; (3) the promoter needs to be a hyperplasia-inducing agent and need not be a carcinogen; (4)...the promotion stage is reversible.

In 1954, Berenblum (5) revised the theory of promotion and maintained that rather than stimulating latent tumor cells to divide, the promoting principle effects a delay in maturation of the dormant tumor cells, at the stem cell stage of development, and consequently causes an imbalance between normal division rate and death rate which produces progressive growth and ultimately a self-perpetuating colony of tumor cells. The validity of this modification may be suspect, however, for as Hieger (31) objects:

> ...The relations of cause and effect are almost impossible to disentagle; e.g. the cancer cells may well be regarded as immature simply because they are proliferating too rapidly for maturation to take place. Berenblum places the two concepts in the reverse order, the neoplastic proliferation being the resultant of the delayed maturation. He is aware of the awkward consequences of this situation, for he says, "Though there is plenty of histological evidence of delayed maturation in tumours, the immaturity of the cells is probably the result of the rapidity of growth of the tumour rather than a causative factor in its development"--a statement which contradicts his earlier thesis.

Immunological

On this molecular level, two theories of carcinogenic mechanisms remain prominent, the one immunological, the other concerned with protein-binding.

The immunological basis of carcinogenesis was first enunciated in 1954 by Green (25) and has been modified and expanded since that time. The initial stimulus to this line of study were two observations: (1) that chemical carcinogens and related although noncarcinogenic substances could increase transplantation immunity, this is, the normal or isoantigenic

immunity, found in all tissues, normal or cancerous, and consequently, the tumors transplanted in animals treated with such compounds would either be rejected or would regress. (2) That although spontaneous tumors are not affected by chemical carcinogens and were originally thought not to produce isoantigens, cancer tissue in general is more easily transplantable than normal tissue. From this point was developed the concept of antigenic deficiency as a feature of the cancer cell. This tumor-inhibiting property of carcinogenic hydrocarbons was found to be not the result of a general depression of somatic growth but rather a specific effect on the transplanted tumor itself. The TI effect was completely abolished by cortisone, and the compounds could elicit a definite proliferation of plasma cells. Green's explanation of this phenomenon involves the hypothesis that the carcinogen binds with tissue protein, forming an auto-antigen which behaves like an isoantigen. Direct attempts to demonstrate specific antibody in carcinogen-treated animals have been unsuccessful. The evidence of immune reactions during carcinogenesis is preponderantly indirect (26). It has been established that cortisone completely abolishes the TI action, that splenic and lymph node pulp from carcinogen-treated animals has an antitumoral effect when mixed with a rat-timor inoculum, and that carcinogenesis is not obtained in tissue culture, indicating that the carcinogen needs organized tissues with an reticulo-endothelial system in which to operate. More direct proof is provided by the experiments of the Millers (52) to be discussed in more detail later, on the binding of liver proteins by azo-dyes, and the antigenicity of such bound proteins, and by the appearance of lipid-reacting antibodies, hemagglutinins and antibody globulin in rabbit serum after skin-painting with carcinogens. Evidence for antigenic loss in tumor cells is also chiefly indirect. According to Green (26),

the cephalin phosphatide in tumor phospholipid exhibits a tumor-enhancing quality and is able to "break down homologous resistance to transplanted tumors and strongly augment growth when the tumor is being restrained by immune forces....it is postulated that during carcinogenesis cells richer in cephalin lipoprotein progressively emerge...(and)...when the cephalin-lecithin lipoprotein ratio, possibly in the cell membrane, has reached a critical point malignancy automatically supervenes." In addition tumor hemagglutinins are also cephalin-containing lipoproteins which seem to be increased in tumors as compared with normal tissues. More direct proof of the loss of tissue specific antigen comes from the work of Weiler (65) who studied two systems: the dimethylaminoazobenzene-induced primary hepatoma of the rat and the stilbestrol-induced kidney carcinoma of the male golden hamster. He prepared liver- and kidney-specific antisera as reagents with hepatoma cytoplasmic particles and kidney carcinoma particles in complement fixation tests. In neither case could he show tissue-specific antigenic activity in the tumors, and concluded that the tissue-specific antigen is "lost, or in some way masked, or decreased in concentration to such a level as to be undetectable by sensitive serological tests." What the effect of such loss on the cancer cell could mean is very indefinite, since the function of the tissue-specific antigen in the normal cell is unknown. At any rate, he has proved that the loss of antigen is <u>not</u> secondary to tumor growth, but rather precedes the occurrence of carcinomata. It is apparent that much more definitive work needs to be done to increase the validity of an immunological theory of carcinogenesis, but it remains one of the more cogent concepts in a highly speculative field.

Protein-Binding

Related to immunology is the theory correlating hydrocarbon carcinogenesis with protein-binding. Hepatocarcinogenic azo-amino dyes were bound to liver proteins (Miller and Miller, 1947). The major fraction of the dye was in the slow-moving fraction of the soluble proteins and none in liver tumors themselves. The same phenomenon occurred with 2-acetylaminofluorene, and 3,4-benzpyrene was bound with the epidermal proteins of mouse skin. In Heidelberger's (28) words:

> It may be stated emphatically at the outset that the binding of chemical carcinogens to the proteins of the susceptible tissue has been found in every case thus far investigated, although the binding of some non-carcinogenic compounds has been found. Thus it appears that the binding of chemical carcinogens to tissue proteins is obligatory for the initiation of cancer.

The Millers are not quite so positive and feel only that such facts "offer support for the hypothesis that the dyes induce neoplastic changes through the gradual deletion of key proteins essential for the control of growth." Heidelberger and his co-workers (28) studied the binding of carbon-14 labelled hydrocarbons to the soluble and particulate proteins of mouse skin after a single topical application and concurred in the following set of conclusions: (1) Binding is through true covalent bonds as seen after treatment and solubilization with pepsin; (2) For binding to occur, the carcinogen must be an intact fully aromatic ring system; (3) Binding takes place through the K region, involves an addition reaction and is subject to steric hindrance; (4) Binding through the L region takes place under some circumstances. He supports the deletion theory and explains that with the loss of certain enzymes (proteins), in the course of cell division that is part of promotion, some daughter cells are produced that lack growth control from the enzyme system--these are the cancer cells.

Electronic

The final theory to be considered relates the electronic configuration of polycyclic hydrocarbons to carcinogenesis. In an extremely technical review involving those concepts so popular with the physical chemist--bond energy, resonance, molecular orbitals and quantum mechanics --Coulson (14) discusses:

> The claim...that there is a certain region, the K-region, in a condensed polycyclic molecule which, by virtue of a high concentration of pi electrons, is able to possess carcinogenic power. The various arguments have all accepted the origin of this power as lying in the pi-electron distribution, but they have differed in the particular combination of derived quantities (bond order, net charge, free valence, energy of excitation, resonance energy) with which a correlation is sought.

He goes on to enumerate four significant factors which of necessity must have considerable influence on how the theory is to be evaluated. First: Pi electrons may not be so exclusively important as previously thought, but may interchange with the ordinary sigma-bond electrons. Second: The relationship of charge and carcinogenesis does not necessarily imply that one determines the other. Rather, both may be due to some more fundamental factor. Third: While the relationship has been found with many compounds, such as the substituted methylbenzanthracenes, it has not been shown with different unsubstituted hydrocarbons. And fourth: The calculations made on bond orders and the like are not very precise and are based on such uncertain foundations that Coulson is forced to say: "It looks therefore as if we are obliged to abandon any hope of a clear-cut description of the relation between electronic configuration and carcinogenesis." Yet in the following paragraphs he maintains:

> There seems to be hardly any reasonable doubt that the total charge on the K-region, coupled with a relatively high bond order, plays a significant part in the activity

> of the carcinogen. The fact that there are some serious failures in this correlation suggests that there may be two or more ways in which the carcinogenicity is shown, or that there are two or more stages in the complete phenomenon, and our K-region analysis deals with only one of these stages--the remaining stages may be governed by an entirely different index, corresponding to an entirely different mechanism.

The Pullmans (58) too, support an electronic theory of carcinogenesis and believe that "the mechanism of action of carcinogenic molecules, at least at one of its stages, a chemical reaction between the carcinogen and a cellular receiver. The reaction probably consists in the formation of an addition product or complex" through the K-region of the carcinogen. The cellular receiver is most likely electrophilic. In addition, for the K-region to develop its carcinogenic potential, the compound must not have an active L-region, since a reaction at the L-region disrupts the molecule, and the aromatic properties are lost. Whether these in vitro reactions do actually occur in carcinogenesis is of fundamental importance. Evidence in favor of such in vivo reactions comes from the arguments of Boyland (11). The metabolic oxidation of carcinogenic hydrocarbons leads through the dehydration of intermediate dehydroxydihydro derivatives to hydroxy derivatives. This transformation however takes place at the region of secondary reactivity, nearly always at positions adjacent to the K-region. Boyland hypothesized that metabolic perhydroxylation takes place at secondary positions because the reactive K bond is already engaged in a different reaction with the cell.

Further attempts to characterize chemical carcinogens by their electronic properties has not been wanting. Polycyclic hydrocarbons form complexes with a variety of substances acting as electron-acceptors and thus producing relatively stable, highly-colored compounds called "charge-transfer" complexes (12). Arcas, et al. (2) state that the stability of

these pi-complexes is enhanced by hydrogen bonds and van der Waals forces, and that such complexes are of a "sandwich" type with their components lying in parallel layers. Mason (48) believes that carcinogenic activity is proportional to the capacity to form complexes with routine electron-acceptors such as picric acid. Huggins and Yang (35) postulate that the co-existence of strong pi-complex-forming properties and a certain geometric pattern similar to the growth-promoting steroids are essential characteristics of compounds which produce mammary cancer.

Buu-Hoï (12) is less enthusiastic and objects to the emphasis placed on complex-formation for two reasons: (1) Complex-building capacity is not an absolute property, but is dependent on the nature of the proposed partner. He and Jacquignom (13) studied a large number of electron acceptors toward both carcinogenic and non-carcinogenic aromatic hydrocarbons and heterocyclic analogs and found that if enough compounds are examined, there is no correlation between biological activity and this particular chemical property. (2) Buu-Hoï (12) also found that after transition from hydrocarbon to functional derivative occurs, the capacity to form charge-transfer complexes sharply decreases, but carcinogenic activity increases. He believes it is probable that complex formation is due more to van der Waals forces and hydrogen bonds than to any real transfer of charge.

CONCLUSIONS AND SUMMARY

The concentration of 3-methylcholanthrene (3-MC) following intraperitoneal administration was determined spectrophotometrically in benzene extracts of the mammary fat pads and interscapular fat pads of female mice. The concentration of 3-MC when varying dosages of the carcinogen were given in sesame oil was fourth mammary fat pad < third mammary fat pad < interscapular brown fat. As the 3-MC dosages increased lineally, the tissue concentration of 3-MC increased nonlineally when examined 7 hours after injection. The peak concentration occurred 10 hours after the intraperitoneal injection of 30 mg. of carcinogen. Clearance of 3-MC from mammary fat pads, which had been isologously transplanted from donor mice 9 hours after injection of 30 mg. of 3-MC, was extremely slow. The tissue concentration 10 days after transplantation was still slightly higher than that of the controls. The carcinogen has a greater affinity for the mammary parenchyma than for mammary fat.

Chemical carcinogens and their possible mode of action have been discussed, along with their ability to induce mammary cancer. Factors influencing mammary carcinoma induction have been evaluated, and the theories proposed for the mechanism of action of chemical carcinogens reviewed.

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